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Nodulation-Dependent Communities of Culturable Bacterial Endophytes from Stems of Field-Grown Soybeans

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Endophytic bacteria (247 isolates) were randomly isolated from surface-sterilized stems of non-nodulated (Nod^-), wild-type nodulated (Nod^+), and hypernodulated (Nod^{++}) soybeans (*Glycine max* [L.] Merr) on three agar media (R2A, nutrient agar, and potato dextrose agar). Their diversity was compared on the basis of 16S rRNA gene sequences. The phylogenetic composition depended on the soybean nodulation phenotype, although diversity indexes were not correlated with nodulation phenotype. The most abundant phylum throughout soybean lines tested was *Proteobacteria* (58–79%). *Gammaproteobacteria* was the dominant class (21–72%) with a group of *Pseudomonas* sp. significantly abundant in Nod^+ soybeans. A high abundance of *Alphaproteobacteria* was observed in Nod^- soybeans, which was explained by the increase in bacterial isolates of the families *Rhizobiaceae* and *Sphingomonadaceae*. A far greater abundance of *Firmicutes* was observed in Nod^- and Nod^{++} mutant soybeans than in Nod^+ soybeans. An impact of culture media on the diversity of isolated endophytic bacteria was also observed: The highest diversity indexes were obtained on the R2A medium, which enabled us to access *Alphaproteobacteria* and other phyla more frequently. The above results indicated that the extent of nodulation changes the phylogenetic composition of culturable bacterial endophytes in soybean stems.

Key words: bacterial diversity, endophyte, legume, nodulation, soybean

Legumes, including soybeans, have been used to analyze the genetic requirements for rhizobial and mycorrhizal interactions in plants. It is well known that the signals involved in both nodulation and mycorrhization overlap in a common signaling pathway, leading to successful symbioses (31, 32). Leguminous plants are also known to regulate the degree of nodulation and mycorrhization of roots by rhizobia and mycorrhizae, respectively (3, 28). This autoregulatory mechanism occurs through long-distance signaling between the shoot and root (30). To date, several studies of soybean-associated microbial communities have been reported (13, 20, 33, 35). However, whether bacterial endophytes use systems similar to those used by rhizobia and mycorrhizal fungi in their associations is largely unknown (32).

Recently, it was shown that the wild-type and symbiosis-defective mutants of the model legume *Medicago truncatula* possess different bacterial community structures and that certain bacteria preferentially associate with mycorrhized roots (29). This example indicates that genetic alteration in the nodulation/mycorrhization signaling pathways can in turn alter the accompanying plant microflora, aside from rhizobia and mycorrhizae. Recently, by using culture-independent methods, we also demonstrated that the nodulation phenotype of soybeans has a marked impact on both the bacterial and fungal diversity in the rhizosphere (15, 16). However, despite the importance of shoot-associated bacteria, which can affect the stress resistance, growth, and development of plants (22–24, 36, 40, 45), our analysis of stem-associated microbes failed because of serious interference by plant DNA.

The aim of the present study was to use a culture-based community analysis to examine the impacts of nodulation phenotype on the diversity of endophytic bacteria in the stems of field-grown soybeans. A parental nodulating soybean and non-nodulating and hypernodulating mutants from the parental line were grown for the community analyses. By using 247 bacterial isolates on three different media, the relationships between the plant genotype and the phylogenetic composition of bacterial endophytes were examined by conducting a clustering analysis of 16S rRNA gene sequences and principal component analysis (PCA) of phylogenetic composition.

Materials and Methods

Plant materials

The plant samples included the parental cultivar Enrei (wild-type nodulating; Nod^+), En1314 and En1282 (non-nodulating mutants derived from Enrei; Nod^-) (10), and En6500 (1) and Sakukei 4 (27) (hypernodulating mutants derived from Enrei; Nod^{++}). The seeds were planted on 31 May 2006 in an experimental field at Tohoku University (Kashimadai, Miyagi, Japan). The field soil was classified as gray lowland soil (pH[H_2O], 5.9; pH[KCl], 4.3; total carbon, 0.98%; total nitrogen, 0.089%; Truog phosphorus, 69 mg P_2O_5 kg^{-1}).

Isolation of endophytic bacteria

Soybean plants were harvested on 30 August 2006 and immediately transported on ice to the laboratory. Plant growth stages of approximately R6 and V14 were obtained for reproductive and vegetative growth indexes (a pod-filling stage), according to the

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method of Fehr *et al.* (9). Although the soybean cultivar Enrei was nodulated, no nodule was observed in the roots of the non-nodulating mutants En1314 and En1282. The soybean lines En6500 and Sakukei 4 showed a hypernodulating phenotype.

The plants were washed well with tap water. Leaves and roots were removed manually. The stems were cut into 5-cm pieces. The stems (10 g) were dipped and shaken in 70% ethanol for a few seconds, then immersed in 2.5% NaOCl solution for 5 min. After repeated washing with sterilized water, the stems were dipped in an ethanol solution and flamed for surface sterilization. They were next cut slantwise into 1-cm pieces with sterilized razor blades. After repeated washing with sterilized water, the stem pieces were placed on plates of three media (R2A agar [R2A], nutrient agar [NA], and potato dextrose agar [PDA]), all of which were purchased from Difco (Detroit, MI, USA). After incubation of the inoculated plates at 25°C for 5 days (Fig. S1), colonies were chosen randomly. The bacteria were purified by single colony isolation on the same medium. After four to six repetitions of single colony isolation, genomic DNA was prepared as described previously (15).

Sequence analysis of 16S rRNA genes

Nucleotide sequences of 16S rRNA genes were determined using the direct PCR method (7). The sequences (1,000–1,100 nt from the 5' end of the 16S rRNA gene) were aligned by using CLUSTAL X (41). Based on the alignment, a distance matrix was constructed by using the DNADIST program from PHYLIP (ver. 3.66; http://evolution.genetics.washington.edu/phylip.html) with the default parameters. The resulting matrices were used as the input for DOTUR (38) to generate diversity indices and different species richness indicators. The default DOTUR settings were used, with a threshold value of 97% sequence identity for the definition of operational taxonomic units (OTUs). Library coverage was calculated with the nonparametric estimator C (11), as described by Kemp and Aller (18). The reciprocal of Simpson's index ($1/D$) was used as a measure of diversity to evaluate the level of dominance in a community (46). A DOTUR-formatted *.list file was used as the input for SONS (39) to calculate the number of isolates in each OTU for each library or for each medium employed for the bacterial isolation.

The phylogenetic composition of the sequences in each library was evaluated by using the Classifier program of RDP-II release 9.6 with confidence levels of 80% (44). BLASTN (2) was also used to classify the clones and identify the nearest relatives in the GenBank database. Sequences were compared on the phylum to genus levels for all possible pairwise library comparisons using the Library Compare program of RDP-II release 9.6 with confidence levels of 80% (5).

Principal component analysis

PCA was performed by using CANOCO (version 4.5 for Windows, Microcomputer Power, Ithaca, NY, USA) with default settings to generate ordination plots based on the scores of the first two principal components using the data for representative sequences and their abundance.

Phylogenetic analysis

The sequences most closely related to those obtained here were searched for in and downloaded from the Sequence Match program of RDP-II release 9.6 (6). For the phylogenetic analysis, deduced amino acid sequences were aligned using the CLUSTAL W program (42). The neighbor-joining method was used for building the trees (37). The PHYLIP format tree output was applied by using the bootstrapping procedure (8); 1,000 bootstrap trials were used. The trees were constructed with TreeView software (http://taxonomy.zoology.gla.ac.uk/rod/treeview.html).

Accession numbers of nucleotide sequences

Nucleotide sequences of 16S rRNA genes for the bacterial isolate libraries have been deposited in the DDBJ database under accession numbers AB461591–AB461837 (247 entries in Table S1).

Results

Bacterial isolation

Endophytic bacteria from the stems of the five soybean genotypes were randomly isolated by using three media according to morphology, color, and size of colonies (Table S1). The 16S rRNA gene sequences were determined for 247 bacterial isolates. The preliminary construction of a phylogenetic tree based on 16S rRNA gene sequences did not permit us to evaluate their relationships clearly with soybean genotypes and isolation media (data not shown). Thus, a statistical approach was adopted for comparing the microbial diversity and phylogenetic composition; such an approach has been used exclusively for culture-independent clone analyses (4, 17, 19, 26).

Statistical analysis

The respective isolate libraries contained between 12 and 18 OTUs (defined by 97% sequence identity; Table 1) for each plant genotype. When all sequences were combined, 42 different OTUs were observed. The coverage of the libraries—calculated by Good's clone coverage algorithm (11) based on species OTUs—was between 62% and 96%. The potential bacterial species richness was calculated for each library by using the nonparametric estimators Chao1 and ACE (Table 1). Although both indexes fluctuated for the five plant genotypes, they were not dependent on nodulation phenotypes (Nod^-, Nod^+, and Nod^{++}) (Table 1). The Shannon indexes for plant genotypes were similar, ranging between 2.1 and 2.7. The values of Simpson's index for plant genotypes were less than 50, indicating that bacterial composition in all libraries was dominant (46).

The impact of the type of medium on the diversity of bacterial isolates was also examined. The number of OTUs obtained by R2A was clearly higher than that obtained by NA or PDA (Table 1). The highest diversity indexes were obtained for R2A medium, while the lowest were obtained for PDA medium (Table 1).

Phylogenetic composition

Using the RDP Classifier, the sequences were placed into a taxonomic hierarchy. The relative abundance of each of the main phyla was determined at the 80% confidence level (Table 1). Endophytic bacteria in soybean stems were classified into four bacterial phyla, the *Bacteroidetes*, *Firmicutes*, *Actinobacteria*, and *Proteobacteria*. Sequence analysis by RDP Lib Compare revealed the taxonomic distribution to differ significantly between libraries, even at the phylum or class level (Table 1). The most abundant phylum throughout all plant genotypes was the *Proteobacteria* (58–79%; Table 1). A high abundance of *Alphaproteobacteria* was clearly observed for non-nodulated soybeans (Nod^-). The most dominant class (47–72%) was the *Gammaproteobacteria* in all except En1314 (Table 1). *Betaproteobacteria* were a minor component. A high abundance of *Firmicutes* was observed for the mutant soybeans (Nod^- and Nod^{++}) as compared to the wild type, Enrei (Nod^+). These results indicate a potential relationship between nodulation phenotype and the phylo-

Table 1. Statistical analyses of bacterial isolate libraries derived from soybean stems

Library (Nodulation phenotype)	Plant genotype[c]					Medium		
	En1282 (Nod^-)	En1314 (Nod^-)	Enrei (Nod^+)	En6500 (Nod^{++})	Sakukei 4 (Nod^{++})	R2A	NA	PDA
Number of isolates	56	29	68	45	49	109	88	50
Number of OTUs[a]	12	18	14	18	16	34	27	14
Library coverage (%)	94.6	62.1	95.6	82.2	89.8	84.4	84.1	92.0
Diversity indices								
Chao1	12.8	27.2	15.0	22.0	17.4	60.2	41.2	14.5
ACE	13.9	34.1	16.1	29.4	21.1	59.2	56.4	16.1
Shannon Index (*H'*)	2.1	2.7	2.3	2.6	2.4	3.0	2.7	2.2
Simpson's index (1/*D*)	6.9	22.7	9.3	12.3	10.1	17.3	12.3	8.4
Phylogenetic compositions (%)[b]								
Bacteroidetes	3.6	3.4	8.8	6.7	6.1	10.1	4.5	0.0
Firmicutes	10.7	10.3	1.5	11.1	12.2	8.3	8.0	10.0
Actinobacteria	7.1	24.1	19.1	24.4	4.1	13.8	20.5	8.0
Proteobacteria	78.6	62.1	70.6	57.8	77.6	67.9	67.0	82.0
Alphaproteobacteria	23.2	34.5	5.9	4.5	4.1	16.5	10.3	8.0
Betaproteobacteria	0.0	6.9	0.0	6.6	2.0	3.6	1.1	2.0
Gammaproteobacteria	55.4	20.7	64.7	46.7	71.5	47.7	55.7	72.0

[a] Operational taxonomic units were defined at 97% sequence identity by using DOTUR.
[b] Sequences were grouped using the RDP Classifier of the Ribosomal Database Project-II release 9 with a confidence threshold of 80%.
[c] Soybean cultivar Enrei (wild-type nodulating; Nod^+); En1282 and En1314 (non-nodulating mutants derived from Enrei; Nod^-); En6500 and Sakukei 4 (hypernodulating mutants derived from Enrei; Nod^{++}).

genetic composition of endophytic bacteria.

Analysis of diversity based on OTUs

The abundance of 16S rRNA gene sequences in each library was assessed on the basis of the 42 OTUs defined at 97% sequence identity and on the phylogenetic relationships of these OTUs (Fig. 1). Among the 42 OTUs, isolates derived from Enrei accounted for only 15 OTUs. The phylogenetic analysis in the present study also indicated that the bacterial isolates from soybean nodulation mutants (Nod^- and Nod^{++}) were distributed over a wider range of taxonomic groups than were the bacterial isolates from Enrei (Nod^+; Fig. 1, Fig. S2).

The high abundance of *Alphaproteobacteria* in non-nodulated soybeans (Nod^-) was explained by the presence of isolates belonging to the family *Sphingomonadaceae* (AP2, AP3, and AP4 of cluster A in Fig. 1 and Fig. S2). The total number of isolates for *Sphingomonadaceae* was significantly larger for the two Nod^- soybean genotypes than for Nod^+ soybeans ($p<0.01$, Fisher's exact test; Fig. S3). Another interesting observation regarding *Alphaproteobacteria* was the absence in Nod^{++} soybeans of the family *Rhizobiaceae* (AP7, AP8, and AP9 of cluster B in Fig. 1 and Fig. S2), which is one of the general enodophytic bacterial groups (12, 24, 25).

The high abundance of *Firmicutes* in the mutant soybeans (Nod^- and Nod^{++}) was shown to be mainly due to the increased number of isolates for *Bacillus* spp. and *Paenibacillus* spp. (F2 and F5 in Fig. 1), whereas only a single isolate of *Bacillus* spp. (F2 in Fig. 1) was obtained in Enrei (Nod^+). The total number of isolates for *Firmicutes* in Nod^{++} soybeans was significantly larger than that in Nod^+ soybeans ($P<0.05$, Fisher's exact test; data not shown). GP8 was shown to be highly abundant in Enrei, but was scarce or absent in the mutant soybeans (Fig. 1, Fig. S3).

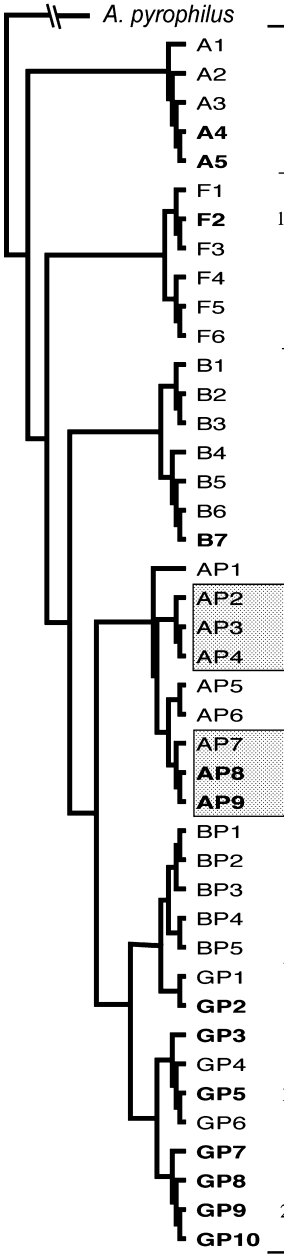
Some groups of OTUs were observed in all plant genotypes and on all media employed (A4, F2, GP2, and GP9 in Fig. 1). Although GP2 was observed in all plant genotypes, this OTU was relatively abundant in Nod^{++} soybeans.

PCA of endophytic bacterial communities

To examine how bacterial diversity was related to plant genotypes, all data were subjected to a PCA (Fig. 2). The PCA results suggested an impact of nodulation phenotype and soybean genotype on the diversity of endophytic bacteria (Fig. 2). The first component (PC1 (36.2%) in Fig. 2) was explained by the differences between nodulation phenotypes, although the second component (PC2 (34.5%) in Fig. 2) showed relatively large variation which was independent of the nodulation phenotypes of soybean lines. The PCA also showed the presence of potential bacterial groups biased toward a nodulation phenotype or a plant genotype. This was particularly clear for some bacterial groups (F4, B7, GP2, GP3, and GP7 in Figs. 1 and 2) towards Nod^{++} soybeans (left side in Fig. 2). On the other hand, the several gray shaded OTUs of *Alphaproteobacteria* belonging to the families *Sphingomonadaceae* (AP2, AP3, AP4) and *Rhizobiaceae* (AP7, AP8, AP9) were biased toward Nod^- soybeans (right side in Fig. 2).

Discussion

Endophytic bacteria were isolated from surface-sterilized stems of soybeans with different nodulation phenotypes by using a conventional cultivation method, and libraries were constructed. To exclude unrelated mutations in soybean mutants, two independent lines derived from Enrei were used for Nod^- and Nod^{++} phenotypes. The communities of bacte-



	Plant genotype (%)					Medium (%)					
	12	13	En	65	S4	R2A	NA	PDA	Closest known species	Acc. No.	Identity (%)
A1		3.4				0.0	1.1	0.0	*Marmoricola* sp.	DQ448721	97.8
A2				4.4		0.9	1.1	0.0	*Arthrobacter* sp.	AY714235	99.6
A3	1.8	3.4				0.9	1.1	0.0	*Labedella kawkjii*	DQ533552	99.9
A4	5.4	17.2	14.7	17.8	4.1	10.1	14.8	8.0	*Microbacterium testaceum*	AF474325	100.0
A5			4.4	2.2		1.8	2.3	0.0	*Microbacterium schleiferi*	Y17237	99.1
F1					2.0	0.9	0.0	0.0	*Exiguobacterium* sp.	DQ083948	99.0
F2	10.7*	6.9	1.5	4.4	2.0	1.8	6.8	8.0	*Bacillus megaterium*	EU723818	99.6
F3					4.1	1.8	0.0	0.0	*Bacillus pumilus*	EU660365	100.0
F4				2.2	4.1	1.8	1.1	0.0	*Paenibacillus* sp.	EU558288	99.2
F5		3.4				0.9	0.0	0.0	*Paenibacillus* sp.	AM162338	99.6
F6				4.4		0.9	0.0	2.0	*Paenibacillus* sp.	DQ358733	99.6
B1				2.2		0.0	1.1	0.0	*Sphingobacterium* sp.	EU483665	99.0
B2		3.4				0.9	0.0	0.0	*Cytophagales* str.	AB022889	97.1
B3					2.0	0.0	1.1	0.0	*Pedobacter rhizospharae*	AM279214	99.0
B4			1.5			0.9	0.0	0.0	*Flavobacterium johnsoniae*	CP000685	99.5
B5			1.5			0.9	0.0	0.0	*Chryseobacterium* sp.	AY468462	98.2
B6	3.6					0.9	1.1	0.0	*Chryseobacterium* sp.	EU373367	99.5
B7			5.9	4.4	4.1	6.4	1.1	0.0	*Chryseobacterium* sp.	AY468475	99.5
AP1				2.2		0.0	1.1	0.0	*Roseomonas genomospecies*	AF533356	97.0
AP2	8.9*	6.9			4.1	4.6	2.3	4.0	*Novosphingobium* sp.	AB360760	99.5
AP3	3.6	6.9				3.7	0.0	0.0	*Sphingomonas* sp.	EU448284	99.0
AP4		3.4				0.9	0.0	0.0	*Sphingomonas* sp.	EU707561	99.4
AP5	1.8			2.2		0.9	1.1	0.0	*Methylobacterium extorquens*	CP000908	99.8
AP6		3.4				0.9	0.0	0.0	*Brevundimonas* sp.	EU567032	100.0
AP7		3.4				0.0	1.1	0.0	*Sinorhizobium* sp.	AM084000	97.4
AP8		6.9	2.9			2.8	1.1	0.0	*Agrobacterium larrymoorei*	EU373312	99.0
AP9	8.9	3.4	2.9			2.8	3.4	4.0	*Rhizobium* sp.	EF540493	99.7
BP1				2.2		0.9	0.0	0.0	*Comamonas* sp.	EU817492	100.0
BP2				4.4		0.0	1.1	2.0	*Delftia acidovorans*	CP000884	100.0
BP3		3.4				0.9	0.0	0.0	*Variovorax ginsengisoli*	AB245358	99.0
BP4					2.0	0.9	0.0	0.0	*Pandoraea* sp.	AY043377	99.9
BP5		3.4				0.9	0.0	0.0	*Ralstonia* sp.	DQ847128	98.9
GP1		3.4				0.0	0.0	2.0	*Luteibactor rhizovicina*	AJ580498	99.0
GP2	1.8	3.4	4.4	13.3	18.4*	11.0	8.0	2.0	*Stenotrophomonas maltophilia*	EU442189	99.8
GP3			5.9	4.4	6.1	1.8	2.3	10.0	*Enterobacter cloacae*	EF025329	98.0
GP4					2.0	0.0	1.1	0.0	*Pseudomonas flectens*	AB021400	96.0
GP5	16.1		17.6	17.8	22.4	11.0	17.0	26.0	*Enterobacter ludwigii*	EF175735	99.0
GP6	7.1	6.9		2.2	10.2	1.8	2.3	16.0	*Pantoea ananatis*	EF602556	99.8
GP7			2.9	4.4	4.1	3.7	2.3	0.0	*Acinetobacter baylyi*	EF178435	100.0
GP8	3.6*	*	19.1	2.2**	**	4.6	10.2	4.0	*Pseudomonas* sp.	EF157292	99.7
GP9	26.8	3.4	11.8	2.2	8.2	12.8	11.4	10.0	*Pseudomonas putida*	EF529517	100.0
GP10		3.4	2.9			0.9	1.1	2.0	*Pseudomonas fluorescens*	AY947533	99.8

Fig. 1. Phylogenetic tree of the 16S rRNA genes of bacterial isolates and their incidence in soybean genotypes and isolation media. The dendrogram (left) indicates the phylogenetic relationships among the 42 representative sequences defined by 97% sequence identity. *Aquifex pyrophilus* (M83548) was used as an outgroup for the dendrogram. Operational taxonomic units (OTUs) were expressed as prefixes derived from phylum identity (A, *Actinobacteria*; F, *Firmicutes*; B, *Bacteroides*; AP, *Alphaproteobacteria*; BP, *Betaproteobacteria*; GP, *Gammaproteobacteria*) with consecutive numbers. The table indicates the abundance (%) of isolates in each soybean genotype or isolation medium and the results of the BLAST search using representative sequences. Blanks indicate no isolate (0%). Enrei is a wild-type, nodulating soybean cultivar (Nod^+). En1282 and En1314 are non-nodulating mutants (Nod^-), while En6500 and Sakukei 4 are hypernodulating mutants (Nod^{++}). Significance in the table was calculated with Fisher's exact test between a mutant soybean and Enrei in respective OTUs. *$p<0.05$. **$p<0.01$. Clusters A and B correspond to *Sphingomonadaceae* and *Rhizobiaceae* in *Alphaproteobacteria*, respectively. Bold indicates that isolates from the mutant soybeans share the same OTU as isolates from Enrei (Nod^+ soybeans).

rial endophytes were partly dependent on the soybean nodulation phenotype (Table 1 and Fig. 1), although the diversity indexes were not correlated with the phenotype (Table 1). The result of the PCA, in which PC1 was in the order of Nod^{++}, Nod^+, and Nod^- phenotypes (Fig. 2), suggests that one of the dominant forces shaping the community structure of stem endophytic bacteria was the nodulation phenotype.

A possible explanation is that the biased phylogenetic composition of endophytic bacteria could be caused by soybean genes for nodulation systems, such as those for the common symbiotic pathway (31) or autoregulation (30) as implied by culture independent-based community analyses with these legume mutants (14, 29). Another possible explanation is that differences in nutrient status gave rise to these community shifts: The supply of nitrogen in Nod^- soybeans is generally deficient compared to that in Nod^+ and Nod^{++}

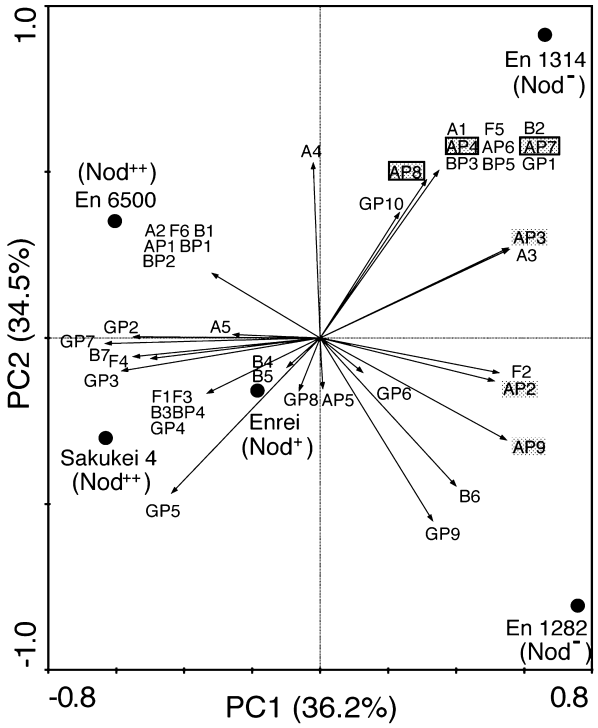


Fig. 2. Biplot with soybean genotypes (●) and bacterial species (arrows) based on a principal component analysis. The names of OTUs correspond to those in Fig. 1. Gray shaded OTUs (AP2, AP3, AP4, AP7, AP8, AP9) belong to the families *Rhizobiaceae* and *Sphingomonadaceae* in the *Alphaproteobacteria*. Enrei is a wild-type nodulating cultivar (Nod^{+}); En1282 and En1314 are non-nodulating mutants (Nod^{-}); and En6500 and Sakukei 4 are hypernodulating mutants (Nod^{++}).

plants.

The diversity of soybean-associated bacteria has been reported with respect to the impact of plant genotype, plant age, plant tissue, plant growth stage sampled (20), or herbicide application (21). In those studies, *Gammaproteobacteria*, such as *Enterobacter* spp. or *Pseudomonas* spp., was found to be the most dominant phylum in both culture-dependent and -independent community analyses. For endophytic bacteria in soybean stems, Kuklinsky-Sobral *et al.* (20) reported that *Gammaproteobacteria* accounted for up to 85% of all isolates. In the present study, again *Gammaproteobacteria* was the most dominant phylum of endophytic bacteria in stems, but the proportion of *Gammaproteobacteria* in all samples was lower than 85%, ranging from 21% to 72% depending on plant genotype (Table 1).

The types of media employed and the procedures for the surface sterilization of tissues may explain the remarkable differences in phylogenetic composition between the present study and the studies reported by Kuklinsky-Sobral *et al.* (20, 21). R2A medium is used for the isolation of bacteria requiring a low nutrient level, such as *Alphaproteobacteria* (34). Indeed, R2A enabled us to access *Alpha-* and *Betaproteobacteria* and other phyla more frequently (Fig. 1, Table 1). In addition, the sterilization was carried out with a combination of chemical treatment and flaming. This procedure may eliminate residual fractions of epiphytic bacteria and may help to reduce the growth competition for endophytic bacteria on a medium. By comparing the results of the present study with previous reports (14, 43), four phyla (*Bacteroidetes*, *Firmicutes*, *Actinobacteria*, and *Proteobacteria*) and three classes of *Proteobacteria* (*Alpha-*, *Beta-*, and *Gammaproteobacteria*) can be considered the core taxonomic groups for endophytic bacteria in soybean stems.

In our previous study using culture-independent methods with the same set of plant roots, bacterial communities in the rhizosphere could be classified into three groups according to nodulation phenotype as well (15). The PCA for DNA fingerprints showed that non-nodulated soybean roots (Nod^{-}) had a microflora that was more similar to that of hypernodulated roots (Nod^{++}) than to that of wild-type nodulated roots (Nod^{+}). In this study, the PC1 in Fig. 2 indicated that the endophyte flora of culturable bacteria in stems of wild-type nodulated soybeans (Nod^{+}) was more similar to that in stems of hypernodulated soybeans (Nod^{++}) than to that in the stems of non-nodulated soybeans (Nod^{-}). The relatively large variation in PC2 (34.5%) in Fig. 2 may be caused by unknown mutations in host genomes since these mutants were created by chemical mutagenesis. At present, we can not explain the difference between the rhizosphere (15) and culturable stem endophytes. However, a result in common was that nodulation phenotype strongly affected the soybean-associated bacterial communities.

Results of the PCA in the present study also revealed the preference of some bacterial groups for certain host genotypes. In the cases of F4, B7, GP2, GP3, and GP7 in Fig. 1, the results imply that these bacteria require a plant receptor such as *NFR1/5* to infect soybeans (32) and also the bacterial population needs to be regulated through the autoregulatory systems for nodule formation (30). For some *Alphaproteobacteria* (AP2, AP3, AP4, AP7, AP8, and AP9 in Fig. 1), their distribution may simply reflect the nitrogen nutrient status of hosts.

Our results also indicated that, by introducing multiphasic analyses such as the clustering analysis and PCA in conjunction with the concept of OTUs, conventional culture-based community analyses could provide more useful information on plant-microbe interactions than had been previously thought. In addition, it is possible to test several hypotheses for positive and negative interactions of specific culturable endophytes in the presence or absence of (brady)rhizobia. Such experiments would facilitate our understanding of the molecular mechanisms of legume-endophyte interactions.

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